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Minireview

Fusing proteins as an approach to study bioenergetic enzymes and processes ☆

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ABSTRACT

Fusing proteins is an attractive genetic tool used in several biochemical and biophysical investigations. Within a group of redox proteins, certain fusion constructs appear to provide valuable templates for spectroscopy with which specific bioenergetic questions can be addressed. Here we briefly summarize three different cases of fusions reported for bacterial cytochrome bc_1 (prokaryotic equivalent of mitochondrial respiratory complex III), a common component of electron transport chains. These fusions were used to study supramolecular organization of enzymatic complexes in bioenergetic membrane, influence of the accessory subunits on the activity and stability of the complex, and molecular mechanism of operation of the enzyme in the context of its dimeric structure. Besides direct connotation to molecular bioenergetics, these fusions also appeared interesting from the protein design, biogenesis, and assembly points of view. This article is part of a Special Issue entitled: 17th European Bioenergetics Conference (EBEC 2012).

1. Introduction

A genetic technique of fusing proteins has traditionally been used in several biochemical and biophysical investigations for variety of purposes. Examples of applications include studies on topographical arrangement of membranous proteins [1], integration of proteins into membranes [2,3], function of enzymes [4,5], interactions between proteins [6], developing protocols for protein isolation by chromatographic methods [7], or detection by microscopic techniques [8,9].

Those types of applications also concern a group of redox active proteins. For example, topographical arrangement of PetL in the cytochrome b_6f complex was studied by fusing PetL with subunit IV before the crystal structure of this complex was known [10]. The fusions with alkaline phosphatase were used to examine the membrane topology of the L-subunit of the photosynthetic reaction center and of the cytochrome *b* subunit of cytochrome bc_1 from *Rhodobacter sphaeroides* [11], while fusions with β-lactamase showed topology of subunit I of the cytochrome *bd* quinol oxidase from *Escherichia coli* [12]. Similar types of experiments aiming at predicting topography can also be found in Refs. [13,14]. One more example includes a model system in *E. coli* with an employment of the fusion of the cytochrome b_6 subunit to maltose binding protein to investigate the incorporation of this subunit into the membrane [15].

In the group of redox proteins, an additional application of the approach of fusing proteins is associated with investigating the function of those proteins. This means designing such types of fusion constructs that would enable addressing specific questions related to electron transfer processes. For example, by studying electron transfer within the chimeric protein containing two, otherwise freely diffusible, redox protein partners (such as in the case of cytochrome b_5 fused to truncated form of NADH-cytochrome b_5 reductase [16]) one might get insights into the mechanisms of protein–protein interactions in the context of inter-protein electron transfer.

To illustrate attractiveness of this approach in investigating various aspects of engineering of bioenergetic enzymes and energy conversion systems, we briefly summarize here three cases of fusions reported for bacterial cytochrome bc_1 (prokaryotic equivalent of mitochondrial respiratory complex III), a common component of electron transport chains. At the root of those studies lies a remarkable structural plasticity of this enzyme which has proven itself capable of tolerating various structural constraints imposed by different types of engineered fusion constructs. In one case, cytochrome bc_1 was fused with its electron carrier protein partner to study supramolecular organization of enzymatic complexes in bioenergetic membrane [17,18]. In another case, two different subunits of the complex were fused to study influence of the accessory subunits on the activity and stability of the complex [19]. In yet another case, the two identical subunits of dimeric complex were fused to study mechanism of operation of the dimer [20–22].

Abbreviations: FeS, 2 iron–2 sulfur Rieske cluster; *Rb.*, *Rhodobacter*; B–B complex, cytochrome bc_1-like complex with two cytochromes *b* fused into one subunit; cytochrome bb, a fusion protein containing two cytochrome *b* subunits connected together by a linker peptide

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2. Constraining diffusion to study supramolecular organization

Biological energy conversion systems are built of multi-subunit membranous protein complexes connected functionally by diffusible components that form redox pools in the membrane (quinone molecules) and in the intermembrane space (cytochromes or other water soluble electron carriers). Diffusion-coupled reactions that link the membranous and water-soluble components are an early idea [23] reinforced by the observation that the lifetime of the complexes formed between the two interacting proteins is very short (less than 400 ns at the physiological ionic strength) [24] and that a dipole moment facilitates collisions to reach a proper configuration for electron transfer [25]. However, the supramolecular organization of the complexes within the membrane is under debate [26–32]. One possibility is that the complexes are more or less randomly distributed throughout the membrane and diffusible components have unrestricted freedom to move between complexes in two dimensions [23,28,29]. Another possibility is that the complexes form larger macromolecular structures with a confined pool of diffusible components [30,32].

As the membranous proteins can, in principle, diffuse independently from one another within the lipid bilayer, investigating the functional status of the macromolecular multi-complex structures from the kinetic point of view presents an experimental challenge. In this context, fusing two redox protein partners offers an attractive solution: one might get effective means to constrain diffusion in a controlled manner to study implications of those constrains on the functionality of electron transfer system. This was accomplished in the cells of purple bacterium *Rb. capsulatus* by fusing cytochrome bc_1 with its redox partner, membrane-anchored cytochrome c_y (Fig. 1A) [17,18]. This cytochrome transfers electrons from cytochrome bc_1 to photosynthetic reaction center sustaining the simplest photosynthetic cyclic electron transfer system (even in the absence of the major periplasmic electron carrier, water-soluble cytochrome c_2) [33–35], thus fusing it with cytochrome bc_1 provided effective means to estimate what was the minimal distance to which cytochrome c_y must move away from cytochrome bc_1 to be able to interact with reaction center and efficiently transfer electrons to it.

The fusion protein complex (cytochrome bc_1–c_y) was constructed by connecting the C-terminus of the cytochrome c_1 subunit of cytochrome bc_1 (which protrudes from the cytoplasmic side of the membrane as the ending part following the transmembranous α-helix anchoring the heme c_1-containing water soluble domain) with the N-terminus of cytochrome c_y (which also protrudes from the cytoplasmic side and continues into the transmembranous α-helix anchoring the heme *c*-containing water soluble domain) [17]. The studies used a series of cytochrome c_y which differed in length in the neck region connecting the heme-containing head domain of cytochrome c_y with its membranous anchor (Table 1). This way, the cytochrome bc_1–c_y fusion served as a "molecular ruler" with which the various distances between the photosynthetic electron transfer system components estimated from the structural constrains imposed by the fusion protein were probed for electron transfer functionality [18].

The shortest and still functional cytochrome c_y linker was found to be about 45 amino-acids long, which implicated that the minimal distance allowed between the cytochrome bc_1–c_y and the reaction center and their surrounding light harvesting complexes can be very short (within 100 Å, necessary for the soluble domain of cytochrome c_y to reach the central part of the L and M subunits of the reaction center and the cytochrome c_1 domain of cytochrome bc_1–c_y fusion complex). This distance range corresponds to reaction center — light harvesting complexes and the cytochrome bc_1 complexes being very close (next) to each other in the membrane. This distance is inconsistent with a model separating spatially the location of the reaction center dimers from cytochrome bc_1 in chromatophore vesicles [36].

The results obtained with the cytochrome bc_1–c_y fusion provided kinetic picture consistent with a notion that individual membranous enzymatic components may form larger structural complexes in bioenergetic membrane. It remains to be seen whether this type of arrangement exists in natural membrane, and if so with what proportion to other possible arrangements (i.e., when complexes are more or less clearly separated apart).

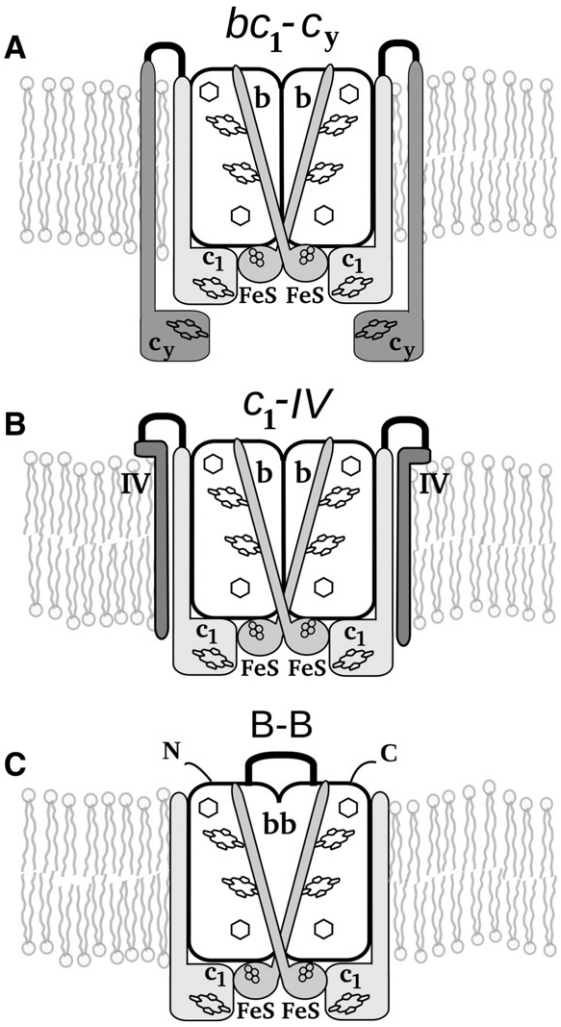

Fig. 1. A schematic drawing of various cytochrome bc_1 fusion proteins. (A) *Rb. capsulatus* cytochrome bc_1–c_y complex (created by a fusion of cytochrome c_1 with membrane-anchored cytochrome c_y), (B) *Rb. sphaeroides* cytochrome c_1-*IV* fusion protein, (C) B–B complex of *Rb. capsulatus* containing fused two cytochrome *b* subunits in the dimer (bb). The black thick lines indicate the position of linkers. b, c_1, FeS, IV, denote subunits of cytochrome bc_1; c_y denotes cytochrome c_y.

3. Improving stability to study structure and assembly

In addition to the three subunits of the catalytic core (cytochrome *b*, cytochrome c_1 and the FeS subunit), several cytochrome bc_1 complexes contain supernumerary subunits with no redox prosthetic groups [37,38]. The number of these subunits may vary: mitochondrial cytochrome bc_1 contains as much as 7 or 8 accessory subunits, while some bacterial species (such as *Rb. sphaeroides*) contains just one accessory subunit (termed subunit IV). These subunits do not participate in the reactions of the catalytic cycle and, in general, their function is less clear comparing to our knowledge on the catalytic subunits. This, in particular, concerns the subunit IV of bacterial cytochrome bc_1 present in some bacterial species (e.g., the above mentioned *Rb. sphaeroides*), but absent in other species (e.g., *Rb. capsulatus*) [39,40]. This small protein, consisting of just one transmembrane α-helix and a loop region at the cytoplasmic side of the membrane [41], does not appear essential for the

Table 1
Selected properties of complexes containing fused subunits of cytochrome bc_1.

Name of fusion protein	Linker length[a]	Connected proteins	Assembly of fusion protein	Enzymatic activity	Photosynthetic growth	References
bc_1–c_y	2	cyt c_1–cyt c_y	+	+	+	[17,18]
bc_1–c_y Δ^b19	2		+	+	+	
bc_1–c_y Δ^b24	2		+	+	+	
c_1-*IV* [6]	6	cyt c_1–su *IV*	+	+	+	[19]
c_1-*IV* [14]	14		+	+	+	
B–B [3]	3	cyt *b*–cyt *b*	+	+	−	[20,22]
B–B [6]	6		+	+	−	
B–B [9]	9		+	+	−	
B–B [12]	12		+	+	−	
B–B [16]	16		+	+	−	
B–B [20]	20		−	nd[c]	nd	

[a] The length of linker corresponds to amino acid residues changed and/or added within the linker region.
[b] Δ indicates number of amino acids deleted from the neck region connecting head domain of cyt c_y with its membrane anchor.
[c] Not determined.

function of the complex although enhances the enzymatic activity of core subunits [40,42]. It is rather loosely bound to the three-subunit core of the complex and can be lost upon purification to various degrees. It also dissociates from the complex upon crystallization [43].

As an approach to prevent this unwanted effect and obtain the complex with stoichiometric amount of subunit IV, the N-terminus of this subunit was fused to the C-terminus of cytochrome c_1 (Fig. 1B) [19]. In this case, a successful fusion was achieved with a linker made of 6 or 14 glycines (Table 1), and the latter one was chosen for further analysis. The purified cytochrome bc_1 complex containing the cytochrome c_1-subunit IV fusion displayed higher enzymatic activity, comparing to typical wild-type cytochrome bc_1 that contain substoichiometric amounts of subunit IV. Interestingly, the complex with the fusion also appeared to be structurally more stable than the wild-type enzyme, as indicated by higher tolerance to detergent treatment and its higher thermotropic denaturation temperature [19]. Those properties make the engineered fusion protein complex a promising material for X-ray crystallography and other structure–function studies.

4. Breaking symmetry to study catalytic mechanism

Cytochrome bc_1 is a homodimer. At the level of monomer, the universally conserved core consisting of three catalytic subunits embeds two hemes *b*, one heme *c*, and the 2-iron–2-sulfur cluster (FeS) [37,38]. Each monomer has two catalytic quinone oxidation sites, the Q_o (Q_p) and Q_i (Q_n) sites, located on the opposite sides of the membrane. From just the structural point of view, the monomer is equipped with all components necessary to perform the main biological function of cytochrome bc_1 — catalyze the Q cycle [44]. However, some of the unusual structural and biochemical–physical properties of cytochrome bc_1 gave rise to considerations that the enzyme may not only be a functional dimer, but also that the catalytic cycle may depend on more or less severe allosteric control within and/or between the monomers. Those elements include: large-scale motion of the FeS head domain between the catalytic Q_o site and cytochrome c_1 necessary to convey its electron transfer function [45], the intertwined topographical arrangement of the FeS subunit seen in the crystal structures [38] (the FeS head domain interacting with the Q_o site in one monomer has its anchor attached to the other monomer), and the distance between the two hemes *b* in the core of the dimer sufficient to support catalytically relevant electron transfer between monomers [46,47].

In view of those considerations, several different models of cytochrome bc_1 catalytic mechanism have been proposed. The allosteric models share a common assumption that the control of the motion of the FeS head domain is required to synchronize specific phases of the motion with specific events of the catalytic cycle [48–51]. The non-allosteric models, on the other hand, assume that thermodynamic grounds are sufficient to explain kinetic operation of the enzyme which simply seeks to establish redox equilibrium between the substrate redox pools [44,46,52,53].

The major experimental difficulty in examining the functioning of the dimer comes from the fact that the monomers are structurally symmetric, and spectroscopically and electrochemically indistinguishable. Furthermore, until recently the experimental means to break this symmetry for functional studies were not available. Because in bacterial cells, the subunits of both monomers are the products of expression of the same genes, the systems for site-directed mutagenesis allowed only symmetrical modifications of cytochrome bc_1 (i.e., mutations were present in both monomers). However, new approaches described recently overcame this limitation [20,22,54,55]. One of them, described for *Rb. capsulatus* cells, is based on a fusion of two cytochrome *b* subunits within the core of the dimer [20,22].

The fusion was accomplished by connecting the C-terminus of one cytochrome *b* with the N-terminus of the other. To do so the gene encoding cytochrome *b* was extended with the linker peptide sequence followed by the second copy of the same gene containing Strep-tag sequence at its terminus. The successful fusion was achieved using a number of different peptide linkers (Table 1), in each case converting two separate 8-helical cytochromes *b* of the dimer into one 16-helical cytochrome bb that assembled together with other subunits, two cytochromes c_1 and two FeS subunits (the so called B–B complex in Fig. 1C) [20]. Such fusion provided template suitable for modifications directing only one copy of individual mutation into cytochrome bb which corresponded to one mutational change per dimer.

With such system the symmetry of the dimer was broken by introducing strategically positioned point mutations that selectively inactivated individual segments of the dimer. One type of mutation (corresponding to G158W in cytochrome *b*) inactivated the Q_o site, the other (corresponding to H212N) inactivated one of the hemes *b* and thereby also the action of the Q_i site. Various asymmetric combinations of these two mutations exposed all major electron-transfer paths for kinetic testing [21,22]. The experiments revealed fundamental principles of the operation of the dimer establishing that electrons move freely within and between monomers, crossing an electron transfer bridge between two hemes *b* in the core of dimer. The so formed H-shaped electron transfer system distributes electrons between four quinone catalytic sites at the corners of the dimer within the millisecond timescale of catalytic turnover [21,22]. In this system any path connecting the catalytic sites on the opposite sides of the membrane is enzymatically competent with no requirement for allosteric control between the monomers and/or the catalytic sites.

The fusion B–B system provides means for systematic examining of various asymmetric electron transfer paths under a broad range of

experimental conditions. This is highly desirable for functional and mechanistic studies. At the level of membranes, kinetics of light-induced electron transfer on a timescale of milliseconds can be followed in various forms of asymmetrically mutated B–B complexes [22]. But also at the level of isolated forms, the B–B complexes retain high enzymatic activities, which extends an array of possible experiments to, for example, those aiming at comparing the maximum turnover rates of several asymmetrically mutated B–B forms [21]. It can be anticipated that in long term, those types of experiments will not only provide mechanistic insights into the operation of dimeric cytochrome bc_1 but will also help understand the role of its symmetry from physiological perspective.

5. Engineering plasticity of bioenergetics systems

Besides direct connotation to molecular bioenergetics, presented above three examples of studies applying a fusion approach in cytochrome bc_1 appear also interesting from the protein design, biogenesis, and assembly points of view. It should be appreciated that even though in all three cases the topographical alignment made it theoretically possible to fuse the two proteins the way they were fused (both linked ends were on the same side of the membrane and were expected to have significant conformational flexibility), a successful expression of designed constructs must have been challenging for the cells as it required adaptations in many processes including protein import, folding, maturation and assembly.

From the three cases discussed here, perhaps the least demanding for the cells was a fusion of subunit IV to the end of cytochrome c_1, as this represented an addition of mostly just one transmembrane α-helix with no cofactors attached to it. But in the cytochrome c_1–c_y fusion, two membrane-anchored cytochromes were connected, each containing covalently attached heme c_1. This required adaptation both in the process of anchoring to the membrane (which normally occurs with a help of signal sequences) and in the post-translational modification to attach hemes (via sophisticated maturation processes) [56].

In the case of the B–B construct, the additional challenge came from the fact that, unlike in the two other cases where the fusion concerned the peripheral parts of cytochrome bc_1 dimer (because of the location of the membranous anchor and the C terminal portion of cytochrome c_1), fusing two cytochromes *b* targeted the central core of the dimer. The 16-helical cytochrome bb construct not only must have folded in a similar manner in the membrane as the two native 8-helical cytochromes *b* but also must have preserved attachment sites for four hemes *b*, must have formed four quinone catalytic binding sites and must have retained specificity in interactions with other subunits, cytochrome c_1 and the FeS subunit. Thus, from an assembly point of view, the competent versions of B–B complexes come as remarkable examples of the overall structural plasticity of cytochrome bc_1 and its hydrophobic core built by cytochromes *b*.

It should be noted that, apart from clear advantages, the fusion approach has its limitations related in part to the fact that fusion constructs are not native to the cells which creates potential risk of occurrence of unassembled or dysfunctional or improperly incorporated into the membrane complexes/subcomplexes at levels that might compromise further functional analysis. Indeed, such cases were documented for some derivatives of B–B [20,22]. One should also bear in mind that fusion proteins should always be treated as model systems, especially when analyzing their functional properties and interpreting the results (see discussion in Ref. [21]).

Electron transport chains assemble as multi-component systems linked together to perform biological function. As exemplified here, in certain cases individual subunits of the same protein or of two separate but interacting-physiologically proteins can be fused together into larger units within which the overall topography, structure, properties of cofactors and catalytic sites and electron transfer connections are preserved. Perhaps such plasticity and flexibility to tolerate more than one way the individual components are getting assembled together is a general property built in to those systems and important from the evolutionary point of view. For us, it is a bonus offering attractive means to study molecular mechanisms of their operation.

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